

Genetic diversity of endangered *Chondrostoma nasus* in the River Rhine system: Conservation genetics considerations on stocking and reintroduction

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Abstract – Reintroduction, stocking and translocation of freshwater fish are of growing concern given their importance for biodiversity conservation and ecosystem functioning. For successful management and stocking programmes, it is essential to incorporate genetics-based approaches. The nase (*Chondrostoma nasus*) constituted one of the most common fish species in European rivers. Its highly specialised and migratory nature exposed the species to human pressures, and thus, promoted its decline. Current knowledge of the genetic structure of *C. nasus* is considerably limited for Europe as a whole and for Germany specifically. To overcome this lack of information we present original data on *C. nasus* from different tributaries of the River Rhine. We analysed nine microsatellite markers and mtDNA Cytochrome *b* sequences to assess the distribution of genetic diversity and structure of this species across the study area. With the exception of the Lake Constance/Alpine Rhine population, *C. nasus* exhibited high gene flow within the Rhine system, and therefore, limited geographical genetic differences between populations where migration is not prevented by human intervention. The present study provides new insights into the levels of genetic variability of *C. nasus* in the Rhine system, providing useful information for guiding reintroduction and stocking programmes. Population genetic information will improve future preservation and management of this valuable freshwater fish species in Germany and beyond.

Keywords: genetic variability / cytochrome b / gene flow / source stock prioritisation / Cyprinidae

Résumé – Diversité génétique de *Chondrostoma nasus*, une espèce menacée, dans le bassin du Rhin : considérations de génétique de conservation sur l'empoissonnement et la réintroduction. La réintroduction, l'empoissonnement et le transfert de poissons d'eau douce sont de plus en plus préoccupants compte tenu de leur importance pour la conservation de la biodiversité et le fonctionnement des écosystèmes. Pour que les programmes de gestion et de repeuplement soient efficaces, il est essentiel d'intégrer des approches fondées sur la génétique. Le hotu (*Chondrostoma nasus*) constitue l'une des espèces de poissons les plus communes dans les rivières européennes. Sa biologie hautement spécialisée et migratoire a exposé l'espèce aux pressions humaines, et a donc favorisé son déclin. Les connaissances actuelles sur la structure génétique de *C. nasus* sont considérablement limitées pour l'Europe dans son ensemble et pour l'Allemagne en particulier. Pour pallier ce manque d'informations, nous présentons des données originales sur *C. nasus* provenant de différents affluents du Rhin. Nous avons analysé neuf marqueurs microsatellites et des séquences d'ADNmt du cytochrome b pour évaluer la répartition de la diversité génétique et la structure de cette espèce dans la zone d'étude. À l'exception de la population du lac de Constance et du Rhin alpin, *C. nasus* présentait un flux génétique élevé dans le système rhénan et, par conséquent, des différences génétiques géographiques limitées entre les populations où la migration n'est pas empêchée par l'intervention humaine. La présente étude apporte de nouvelles connaissances sur les niveaux de variabilité génétique de *C. nasus* dans le système rhénan, fournissant des informations utiles pour

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orienter les programmes de réintroduction et d'empoissonnement. L'information génétique des populations permettra d'améliorer la préservation et la gestion futures de cette précieuse espèce de poisson d'eau douce en Allemagne et au-delà.

Mots clés : variabilité génétique / cytochrome b / flux génétique / priorisation du stock source / Cyprinidae

1 Introduction

Human interventions in freshwater ecosystems worldwide have affected the abundance and distribution of freshwater organisms with the consequences of causing taxonomic homogenisation by non-native species introduction (Villéger *et al.*, 2011), population declines or even species losses (Vaughn, 2010). With at least 39% of freshwater fish being threatened, they are one of the most severely threatened taxonomic groups being assessed to date (Freyhof and Brooks, 2011). Even though fish restoration practices were realised throughout Germany and other countries, efforts often failed to establish self-sustaining and reproductive populations. Reasons of failure may be that management purposes often rest on private activities, are conducted at a local scale and still show deficits in the compilation, coordination and exchange of knowledge between scientists and managers (Lundmark *et al.*, 2019). For many native freshwater fish species, neither national nor international, genetically informed management strategies exist. However, in Germany there are attempts to collate genetic information of freshwater fish *e.g.* in the AGRDEU database (<https://agrdeu.genres.de/nationales-inventar-aqgr/>) to provide information on the genetic diversity of hatchery strains and wild fish populations for political consulting, management purposes and information exchange. Genetic information is necessary for adopting appropriate conservation strategies, will be of help to maintain biodiversity and to prevent the loss of genetic resources and the eventual erosion of ecosystem function (Brodersen and Seehausen, 2014). Intraspecific diversity within a species may reflect either historical isolation (genetic drift, reinforced by limited gene flow between disconnected populations), patterns of local adaption (natural selection), or both. Either way, recognizing and preserving diversity is substantial for successful conservation on the long term. It is expected to stabilize ecosystem services and serve as a portfolio effect buffering metapopulations against pervasive effects of environmental change and perturbation (Schindler *et al.*, 2010; Piccolo *et al.*, 2018).

Even though the nase (*Chondrostoma nasus* L.) has a wide distribution area and was a common fish species in Europe (Flore and Keckeis, 1998), many populations have declined especially in Germany, Switzerland, Austria, Poland and the Czech Republic (Penáz, 1996). The River Rhine system is the westernmost range limit of the species' natural distribution (Hudson *et al.*, 2014), whereas nase has been introduced to further rivers in France, including the Rhone and Loire rivers. In the Rhine system and its tributaries such as the rivers Lahn, Neckar, Main, Nidda and Kinzig, nase was historically present in high abundances (Dußling and Berg, 2001; Hmuklv and Hessen-Forst Fena, 2014). However, in several surveys of the River Rhine basin in the 1990s, nase occurred only sparsely at low numbers and was considered extinct in the River Lahn (Freyhof, 1997, and cited literature within). In consequence of

the drastic decline in the River Lahn, first attempts to reintroduce the species were implemented. In the years 1995–1997, 154.000 fish were stocked between the upper Lahn, near Kernbach and the middle reaches of the Lahn, near Steeden (Fig. 1). Stocked fish originated from a hatchery stock that derives from the River Nister in the River Rhine system (Fig. 2). Intense monitoring in subsequent years revealed no success (Schwevers and Adam, 1997) and have led to the conclusion that nase was in fact gone extinct in the entire Hessian catchment of the river Lahn. Sporadic and widely scattered encounters ($N=5$ in total) were recorded and interpreted as remnants of specific local stocking efforts. However, no reproduction was detected (Schwevers and Adam, 1997). Only since 2006, fish were recorded five kilometres upstream Steeden in the middle reaches of the Lahn (Fig. 1). Since 2009 nase was caught frequently in the middle reaches (Hübner and Fricke, 2011, 2012, 2014) and a reproductive, self-sustaining population was re-established (Hübner *et al.*, 2016). It remains uncertain whether fish in the middle reaches of the Lahn originated from the first reintroduction activities or from an autochthonous remnant stock of the watershed itself that has survived the collapse years ago and recovered again. However, in the upper River Lahn above Marburg, still no nase population exists. Further reintroduction attempts in the upper River Lahn in the years 2014 and 2015 used the same hatchery stock as in the 1990s, but failed again. Several weirs between the middle and upper reaches of the Lahn exist and may represent insurmountable obstacles for the upstream migration of nase (Fig. 1). In addition, different habitat conditions in the upper River Lahn (*e.g.*, higher discharge, narrower riverbed) and/or improper genetic-based characteristics of the stocked fish could have prevented a re-establishment of nase in the upper Lahn. Since 2017, stocking strategies for the reintroduction of nase in the upper Lahn have been changed as part of the EU-LIFE project "Living Lahn" (Hübner *et al.*, 2017). Since then, juveniles are produced by stripping spawning animals from the middle Lahn itself and the fertilized eggs are then raised up to stock size at a hatchery farm until they are released into the upper Lahn.

Genetic variability patterns are essential for a sustainable management and provide relevant information for future stocking programmes. Genetic information is needed to avoid inbreeding and to preserve the adaptive potential of a species since genetic diversity is linked to biological productivity and resilience to stressors. So far, the population genetic structure of the nase in Germany has been investigated to a very limited extent. To our knowledge, only one population genetics study investigated German nase populations (Hudson *et al.*, 2014). In this context, the aims of the present study were (i) to assess the genetic diversity and variability of the widespread but locally threatened species in its historically native distribution range within the River Rhine system, (ii) to assess gene flow and identify possible distinct genetic populations, (iii) to ascertain

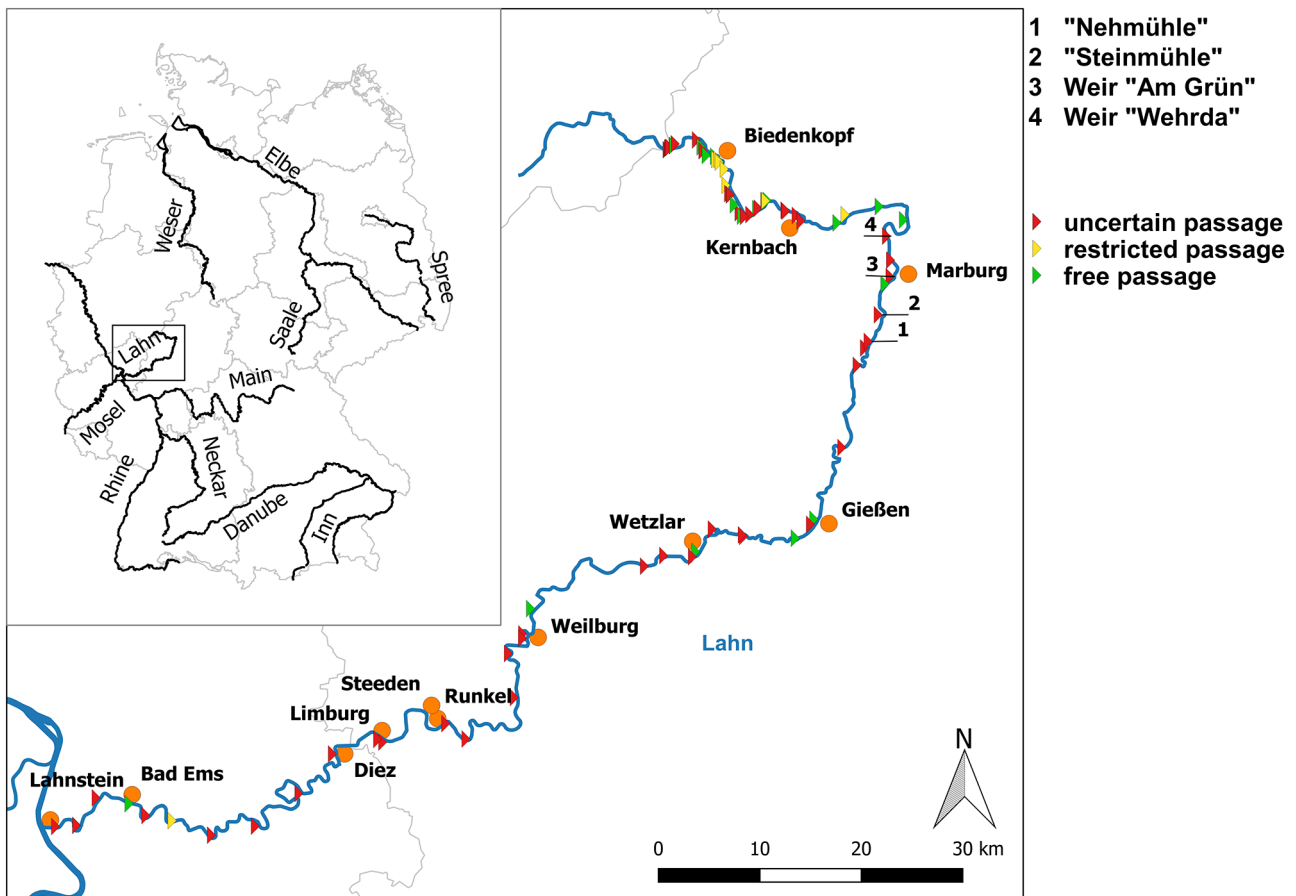


Fig. 1. The course of the River Lahn and its obstacles impeding fish migration. Stocking sites of *Chondrostoma nasus* in the years 1995–1997 and 2014–2015 were located between the upper course (near Kernbach) and the middle course (near Steeden) of the River Lahn.

the origin of the population found in the middle reaches of the River Lahn (stocked hatchery fish or survived autochthonous stock), and (iv) to identify the most suitable stock for future reintroduction of the nase in the upper River Lahn.

Based on genetic information, this study will contribute to improve our knowledge for the development of management strategies for the freshwater specialist *C. nasus* and its declining autochthonous populations in Germany.

2 Material and methods

2.1 Sampling

Chondrostoma nasus individuals were collected from 10 locations in the River Rhine system (Tab. 1, Fig. 2) and from two hatchery stocks. The hatchery_1 stock (H1) was originally established from fish of the river Main near Obernburg, whereas the hatchery_2 stock (H2) was established from fish of the River Nister in 1984 and was used for the reintroduction of *C. nasus* in the River Lahn until 2015. The population named LA (Lahn) in this study originates from the middle reaches of the River Lahn. Several weirs mark the watercourse between the middle and upper River Lahn (Fig. 1) and it is uncertain whether nase is able to pass these upstreamwards. Investigated specimens from the River Murg, Wiese and Dornbirner Ach originate from the study of

Hudson *et al.* (2014) but were reanalysed with a different mtDNA marker in this study. Wild fish were caught by electrofishing. From all fish, fin clips were taken in situ and stored in 96% ethanol at -20°C until further processing. Fish were released at their home locality immediately after sampling.

2.2 Sequence analysis

DNA extraction was performed following a modified (Wetjen *et al.*, 2017, 2020) DNA salt-extraction protocol (Aljanabi and Martinez, 1997). A 1094 bp fragment of the mtDNA cytochrome *b* gene (*Cytb*) was obtained for 154 specimens using the primers Glu-L and Thr-H (Mäkinen and Merilä, 2008). Polymerase chain reactions (PCR) were performed using a Primus 96 Cycler (Peqlab Biotechnologie GmbH, Erlangen, Germany) under the following conditions: Denaturation (2 min, 95°C) was followed by 25 cycles of 30 s at 94°C , 30 s at 54°C and 45 s at 72°C , and a final elongation step (10 min, 72°C). The company SeqIT GmbH & Co.KG (Kaiserslautern, Germany) conducted bidirectional sequencing of the DNA strands. Sequence segments were aligned using the programme Geneious v. 6.1.8 (Biomatters Ltd.). Generated sequences were compared with those available in GenBank using the 'Basic alignment search tool' (BLAST).

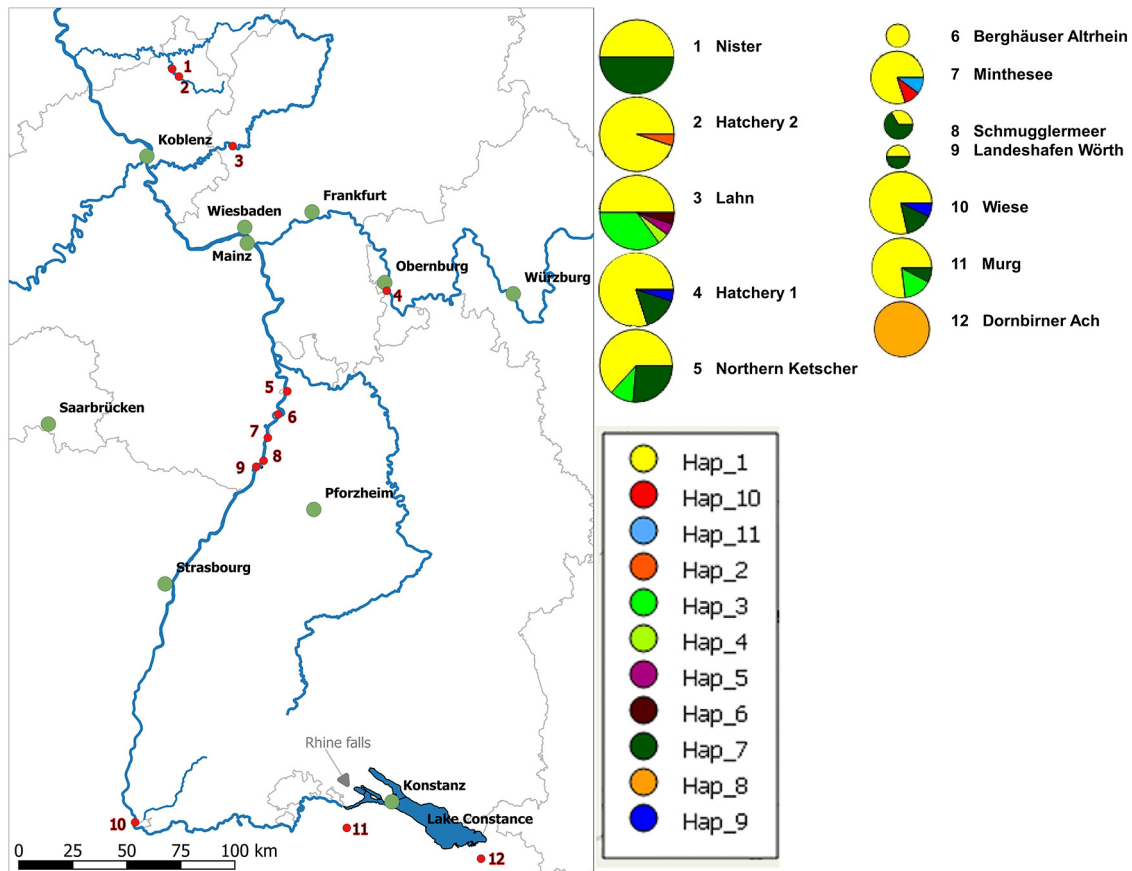


Fig. 2. Sampling locations and observed haplotypes per sampling site of *Chondrostoma nasus* in the River Rhine basin. The size of the circles is proportional to the number of analysed individuals per sampling site.

Table 1. Sampling sites and number of analysed *Chondrostoma nasus* individuals per population (Pop) at the sequence (N_{mtDNA}) and microsatellite (N_{MS}) level in the River Rhine system.

Pop	Sampling site	Sampling year	Geographical coordinates		N_{mtDNA}	N_{MS}
			N	E		
H2	Hatchery_2	2015	—	—	20	50
LA	Lahn	2015	50°24'11.82"	8°8'46.94"	20	42
NI	Nister	2015	50°43'14.58"	7°44'36.44"	20	51
DO	Dornbirner Ach	2008	47°26'11.15"	9°43'09.35"	11	14
WI	Wiese	2008	47°34'53.29"	7°35'31.77"	14	14
MU	Murg	2008	47°34'01.20"	8°53'42.67"	13	13
SM	Schmugglermeer	2017	49° 5'40.39"	8°21'52.28"	3	3
MI	Minthesee	2017	49°11'24.98"	8°23'24.57"	10	10
BA	Berghäuser Altrhein	2017	49°17'17.70"	8°27'18.10"	2	2
LW	Landeshafen Wörth	2017	49° 4'8.86"	8°19'5.79"	2	2
NK	Northern Ketscher	2017	49°23'4.43"	8°30'43.80"	19	43
H1	Hatchery_1	2017	—	—	20	40

2.3 Microsatellite analysis

Nine microsatellite loci were co-amplified for 284 individuals: LC27, LC290 (Vyskocilová *et al.*, 2007), Lsou05,

Lsou08, Lsou21 (Muenzel *et al.*, 2007), SarN7F11b, SarN7F8, SarN7G5, SarN7K4 (Mesquita *et al.*, 2003). Multiplex-PCRs were performed according to Hudson *et al.* (2014) with slight modifications resulting in a reduction of the used primer

concentrations. Fragment analysis was performed on a Beckman Coulter CEQ 8000 eight capillary sequencer. Loci were scored using the software CEQ SYSTEM v. 9 (Beckman Coulter).

2.4 Data analysis

A median-joining (MJ) network (Bandelt *et al.*, 1999) of the generated mtDNA sequences was constructed using the programme PopART (Leigh and Bryant, 2015). To obtain meaningful, unimpaird results, fish of the populations BA, LW and SM were only included in the MJ Network but excluded from any further analyses as only 2–3 individuals were available from these locations (Tab. 1). Haplotype (h) and nucleotide diversity (π) indices were estimated in DNAsp v.5.10.1. True diversity (D_{mtDNA}) was calculated as the effective number of haplotypes per population (Jost, 2010). To analyse the demographic history and evidence of population expansion at the sequence level, Tajima's D (Tajima, 1989), Fu's F_S (Fu, 1996), and Ramos-Onsins and Rozas R_2 (Ramos-Onsins and Rozas, 2002) statistics of neutrality were estimated in DNAsp v. 5.10.1. Significance was assessed from 1000 coalescent simulations.

MICRO-CHECKER v. 2.2.3 (Van Oosterhoudt *et al.*, 2004) was used to test for the presence of null alleles, allele dropouts and scoring errors in the microsatellite dataset. Observed (H_O) and expected (H_E) heterozygosity, deviations from Hardy–Weinberg equilibrium (HWE), linkage equilibrium (LD), and the fixation index (F) were assessed using GenAlEx v.6.5 (Peakall and Smouse, 2012). Values of a fixation index close to zero are expected under random mating, whereas substantial positive values are indicative of inbreeding. True diversity (D_{MS}) was estimated as the effective number of alleles per population (Jost, 2010). Allelic richness (A_R , *i.e.* Petit *et al.*, 1998) and private allelic richness (P_{AR} , *i.e.* Kalinowski, 2004) were calculated using rarefaction in HP-RARE (Kalinowski, 2005). Effective population size (N_e) was estimated using NeEstimator v.2.1 (Waples *et al.*, 2014) and the number of migrants (N_m) using GENEPOP v. 1.2 (Raymond and Rousset, 1995). Additionally, first generation migrants (F_0) were identified in GeneClass2 (Piry *et al.*, 2004) to evaluate migrants influence on genetic differentiation. To account for unsampled populations and maximising the analyses power, we used two test statistics ($L_{\text{home}}/L_{\text{max}}$ and L_{home}) to compute the likelihood of migrant detection (L) at the microsatellite level (Paetkau *et al.*, 2004). Furthermore, a self-assignment computation was conducted for the individual genotypes using a frequency-based method with an assignment threshold of 0.05 in GeneClass2.

A Bayesian, Markov Chain Monte Carlo (MCMC) analysis was conducted to assess the extent to which allelic structures of the sampled populations could be clustered into the same group (K) utilizing the programme STRUCTURE v. 2.3.3 (Pritchard *et al.*, 2000). An admixture model with correlated allele frequencies and prior information about individual populations was used to further shape the analysis. The analysis was run ten times for each K (1–16) with a burn-in period of 50,000 followed by 500,000 MCMC. The most probable K was ascertained following the delta K method (Evanno *et al.*, 2005) using Structure Harvester (Earl and Von Holdt, 2012). The

detection of one cluster would indicate high admixture between populations, whereas different geographic clusters would indicate that populations might have experienced genetic drift leading to genetic differentiation.

In addition, a distance-based principle component analyses (PCoA) was performed with data standardisation for nose populations to visualise the variance in microsatellite allelic structure within the River Rhine basin. PCoA was conducted using GenAlEx v.6.5 (Peakall and Smouse, 2012).

We computed measures of pairwise differentiation represented by the population level fixation indices F_{ST} (microsatellites) and Φ_{ST} (mtDNA sequences). Significance was assessed using 10,000 permutations. Hierarchical analysis of molecular variance (AMOVA) was additionally performed for both marker types to detect differentiation in genetic variability among populations. Both analyses were conducted in Arlequin v. 3.11 (Excoffier *et al.*, 1992).

To assess the effective number of distinct populations, we calculated the between-population component Δ_{ST} for both marker types as described in Jost (2008). To determine the contribution of the individual populations to the total distinctiveness, Δ_{ST} was additionally calculated removing each individual population separately from the entire dataset (including all but one population). This allows for comparing the change in Δ_{ST} when one population is excluded, and hence, estimating its contribution to the overall effective number of distinct populations.

Demographic processes such as expansions and reductions in the effective population size affect genetic diversity. During a bottleneck, characteristic mode-shift distortion in the distribution of allele frequencies can be detected since allelic diversity is supposed to be reduced faster than heterozygosity (Luikart *et al.*, 1998). While low frequency alleles will get lost, the remaining alleles maintain present in intermediate frequencies. Hence, it is expected that the observed heterozygosity is larger than the heterozygosity expected from the observed allele number at mutation-drift equilibrium (Cornuet and Luikart, 1996). We used the programme BOTTLENECK (Piry *et al.*, 1999) to test for recent bottlenecks in individual populations applying the stepwise mutation model (SMM, Kimura and Ohta, 1978) and the two-phase mutation model (70% stepwise mutations, TPM). We used the sign test and the Wilcoxon signed-rank test to assess significant heterozygosity excess (Piry *et al.*, 1999). Significance of multiple comparisons was determined by adjusting p -values for false discovery rates (FDR) following the method of Benjamini and Hochberg (1995).

3 Results

3.1 Haplotype diversity, distribution and demography

In total, 11 different mitochondrial *Cytb* haplotypes were observed in the River Rhine basin (Fig. 2). Haplotypes were differentiated by a maximum of 26 mutational steps (Fig. 3). The haplotype Hap_1 was most common ($N=100$; 64.94%) and present in all populations except the Dornbirner Ach, a tributary of the Alpine Rhine upstream of Lake Constance. The haplotype Hap_1 was already identified in a previous study (Accession number: JQ652366.1) by Dubut *et al.* (2012), whereas the other ten haplotypes were newly identified for

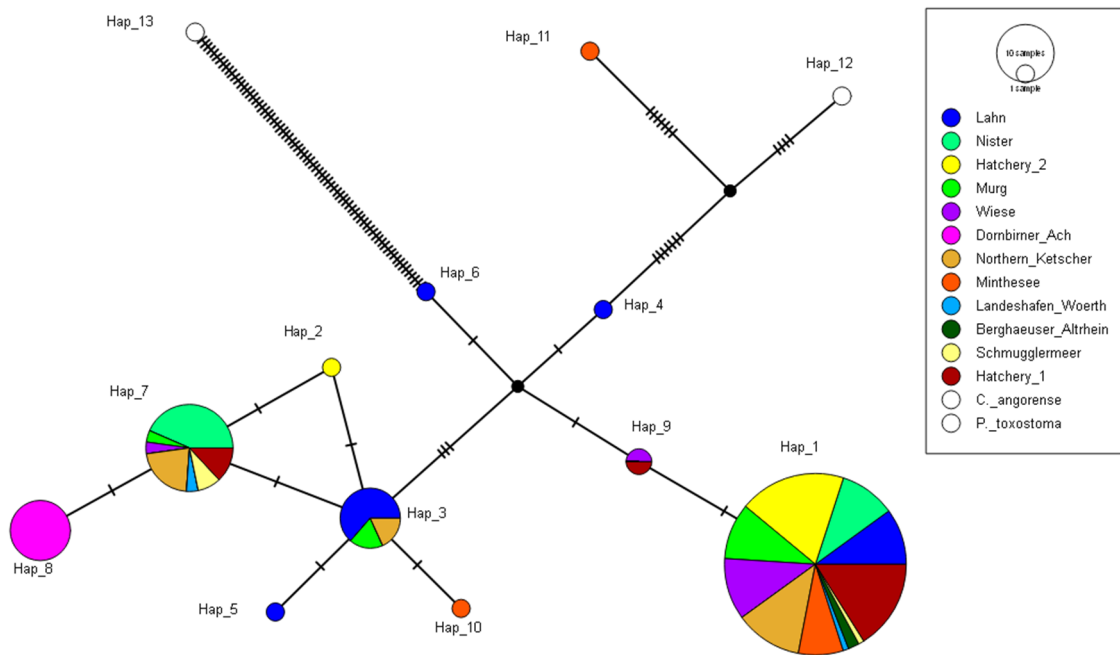


Fig. 3. Median-joining network of 154 individuals of *Chondrostoma nasus* of the River Rhine basin including reference haplotypes of *Chondrostoma angorensis* (Hap_12) and *Parachondrostoma toxostoma* (Hap_13). The size of the circles is proportional to the frequency of fish carrying the respective haplotype. Colours refer to the origin-sampling site. Mutational steps are represented by intermediated dashes. Black dots represent missing haplotypes.

Table 2. Summary of genetic diversities within the investigated *Chondrostoma nasus* populations. For each population (Pop) true diversity (D_{MS}), allelic richness (A_R), private allelic richness (P_{AR}), observed (H_O) and expected heterozygosity (H_E), fixation index (F), true diversity (D_{mtDNA}), number of haplotypes (N_{Hap}), haplotype diversity index (h), number of private haplotypes (P_{Hap}), segregating sites (S) and the nucleotide diversity index (π) is reported.

Pop	D_{MS}	A_{R10}	P_{AR10}	H_O	H_E	F	N_{Hap}	D_{mtDNA}	h	P_{Hap}	S	π
H2	2.97	3.38	0.02	0.663	0.613	-0.087	2	1.11	0.100	1	6	0.001
LA	3.14	4.16	0.42	0.681	0.680	0.006	5	2.88	0.653	3	8	0.003
NI	2.56	3.65	0.13	0.623	0.613	-0.027	2	2.11	0.526	0	6	0.003
DO	2.39	3.63	0.48	0.582	0.565	-0.036	1	1.00	0.000	1	0	0.000
WI	3.00	3.53	0.06	0.667	0.602	-0.086	3	1.63	0.385	0	6	0.002
MU	3.21	3.97	0.21	0.688	0.659	-0.048	3	1.70	0.410	0	6	0.002
MI	3.10	3.99	0.10	0.677	0.637	-0.021	3	1.61	0.378	2	21	0.004
NK	3.13	3.85	0.16	0.680	0.662	-0.024	3	2.22	0.550	0	6	0.003
H1	2.97	3.49	0.26	0.663	0.601	-0.090	3	1.54	0.353	0	6	0.002

nase in this study (deposited in GenBank; Accession numbers: MN431659–MN431668). The highest number of private haplotypes was present in the River Lahn with $P_{Hap}=3$. Diversity ranged from $D_{mtDNA}=1.00$ (DO) to $D_{mtDNA}=2.88$ (LA) at the sequence level (Tab. 2). Fish of the Dornbirner Ach exclusively carried a single haplotype (Hap_8). The haplotype Hap_8 was private for this site and is probably restricted to nase from the Alpine Rhine/Lake Constance basin. Noticeable is the haplotype Hap_11, which was only present in the Minthesee population with one individual. This haplotype was most distantly related to all other haplotypes by at least 15 mutational steps (Fig. 3). It was not found in GenBank but was closest related (98.99% identical; differentiated by 12

mutational steps) to a *Chondrostoma angorensis* haplotype found in the Kizilirmak river, Turkey (Perea *et al.*, 2010; Accession number: HM560078.1).

Neutrality tests revealed no recent population expansion within the studied populations (Tab. 3). Tajima's D were significantly negative only in the hatchery_2 and the Minthesee populations. However, Fu's F_S and R_2 were positive and not significant for both these populations.

3.2 Microsatellite diversity

Data showed no evidence for null alleles, large allelic dropout or scoring errors. Genotype proportions for all

Table 3. Neutrality tests and evidence for recent population bottlenecks using the sign test and the Wilcoxon test of investigated populations (Pop) of *Chondrostoma nasus*. D_T = Tajima's D ; F_S = Fu's F_S ; R_2 = Ramos-Onsins and Rozas R_2 ; TPM = two-phase model; SMM = stepwise mutation model. Significant values are in bold.

Pop	F_S	D_T	R_2	Sign test: Expected and observed no. of loci with heterozygosity excess (probability)		Wilcoxon test (Probability of heterozygote excess)	
				TPM	SMM	TPM	SMM
H2	1.743	-2.056	0.218	4.9/9 (p = 0.004)	5.1/9 (p = 0.006)	0.001	0.001
LA	1.773	0.917	0.167	5.2/2 (p = 0.032)	5.2/2 (p = 0.035)	0.918	0.981
NI	7.629	2.764	0.263	5.1/4 (p = 0.340)	5.2/4 (p = 0.314)	0.410	0.898
DO	—	—	—	5.1/2 (p = 0.038)	5.1/2 (p = 0.037)	0.997	0.997
WI	2.093	-0.391	0.140	5.1/7 (p = 0.171)	5.3/5 (p = 0.553)	0.213	0.455
MU	2.563	0.274	0.166	5.3/7 (p = 0.205)	5.2/6 (p = 0.426)	0.082	0.248
MI	4.481	-1.850	0.216	5.3/4 (p = 0.301)	5.2/3 (p = 0.127)	0.752	0.898
NK	4.537	2.166	0.239	5.2/3 (p = 0.123)	5.2/2 (p = 0.033)	0.820	0.986
H1	2.63	-0.023	0.140	5.2/5 (p = 0.593)	5.2/4 (p = 0.318)	0.065	0.674

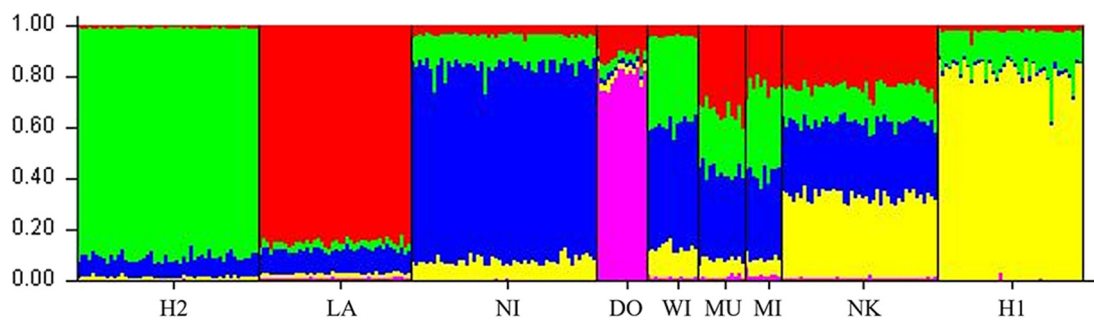


Fig. 4. Bayesian clustering analysis of the investigated *Chondrostoma nasus* populations: H2 = Hatchery_2, LA = Lahn, NI = Nister, DO = Dornbirner Ach, WI = Wiese, MU = Murg, MI = Minthesee, NK = northern Ketscher, H1 = Hatchery_1. Colours refer to the predicted clusters (1–5). Each individual is displayed by a vertical column with the length proportional to the individual's estimated affiliation to the respective cluster.

populations conformed to HWE expectations ($p > 0.05$). Only few loci showed a deviation from HWE in some populations (Lsou05, SarN7G5 in DO; Lsou21, SarN7F8 in MI; LC27, LC290, Lsou08 in NK). No significant linkage disequilibrium between markers was detected. Allelic richness was highest in the Lahn ($A_R = 4.16$) and the Minthesee ($A_R = 3.99$) populations, whereas the hatchery_2 population ($A_R = 3.88$) showed lowest mean allelic richness (Tab. 2). Private allelic richness was highest in the Lahn ($P_{AR} = 0.42$) and Dornbirner Ach ($P_{AR} = 0.48$) populations. True diversity was highest in the Murg ($D_{MS} = 3.21$) and Lahn ($D_{MS} = 3.14$). However, the hatchery_2 stock showed a comparatively lower overall diversity, indicating a slightly reduced genetic diversity in this stock (Tab. 2).

3.3 Gene flow and population structuring

Clustering of samples into population groups via the Bayesian clustering analysis gave the highest probability for $K = 5$ (Fig. 4). One cluster (K1) was formed by the hatchery_2 stock and a second cluster (K2) by the Lahn population. The third cluster (K3) was formed by the hatchery_1 stock and the fourth (K4) by fish from the Dornbirner Ach of the Alpine

Rhine. All remaining populations (Nister, Wiese, Murg, Minthesee, and northern Ketscher) grouped predominantly into the fifth cluster (K5) but displayed high admixture, especially with individuals belonging to the clusters of both hatchery stocks and the Lahn population. We did not find any evidence for gene flow between the Dornbirner Ach and any of the Rhine populations from downstream of the Rhine Falls.

PCoA confirmed the unique genetic signature of the Dornbirner Ach population within the Rhine system (Fig. 5). In addition, the Lahn population and both hatchery stocks (H1 and H2) grouped further apart from the rest of the investigated Rhine populations.

The AMOVA (Tab. 4) revealed that the genetic variance was explained by the variance within populations by 70.39% and between populations by 29.61% at the sequence level. Microsatellites showed also that most variation was explained by the variation within the individual populations (95.45%), whereas only 4.55% of the variation was explained by the variation among the different populations.

Effective number of distinct populations resulted in $\Delta_{ST} = 1.379$ at the mtDNA level and $\Delta_{ST} = 1.226$ at the microsatellite level for the nine populations (Tab. 5). This indicates that we cannot find definite distinct structuring within

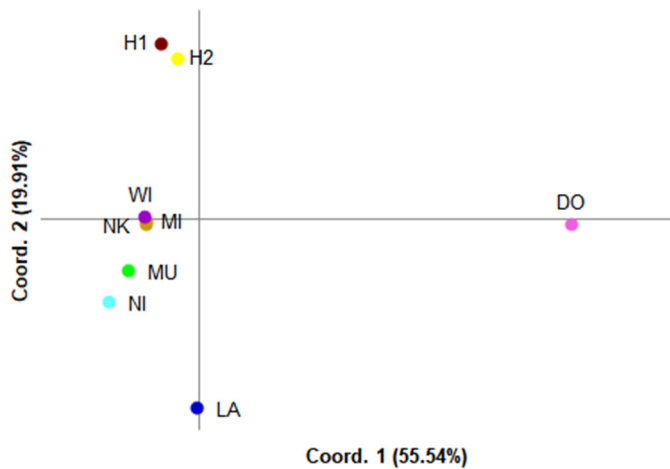


Fig. 5. Plots of the first two axes of a principal component analysis (PCoA) of *Chondrostoma nasus* populations using GenAlEx v. 6.5. Each coloured dot represents one population: H1 = Hatchery_1, H2 = Hatchery_2, NI = Nister, LA = Lahn, NK = northern Ketscher, MI = Minthesee, MU = Murg, WI = Wiese, DO = Dornbirner Ach.

Table 4. Analysis of molecular variance (AMOVA) for the sequence (mtDNA *Cytb*) and microsatellite (MS) data of *Chondrostoma nasus*. Shown is the degree of freedom (df), the sum of squares (Sum of sq.) and the variation in percent for each source of variation.

	Source of Variation	df	Sum of sq.	Variation (%)
mtDNA	Among populations	8	67.25	29.61
	Within populations	138	148.38	70.39
MS	Among populations	8	94.11	4.69
	Within populations	545	1634.82	95.31

Table 5. Change in the effective number of distinct populations (Δ_{ST}) excluding individual populations from the entire dataset (all Pops). Δ_{ST} and the change (difference) are shown for both, the sequence (mtDNA) and microsatellite (MS) data.

	mtDNA		MS	
	Δ_{ST}	difference	Δ_{ST}	difference
All Pops	1.379	—	1.226	—
–MI	1.411	0.032	1.231	0.005
–MU	1.430	0.051	1.235	0.009
–DO	1.104	–0.275	1.181	–0.045
–WI	1.432	0.054	1.222	–0.004
–H1	1.460	0.081	1.228	0.002
–LA	1.349	–0.030	1.212	–0.014
–NK	1.427	0.049	1.243	0.017
–H2	1.461	0.082	1.234	0.008
–NI	1.353	–0.026	1.220	–0.006

the Rhine basin below the Rhine Falls. However, the contribution of the individual populations to the effective number of distinct populations was highest for Dornbirner Ach

(mtDNA Δ_{ST} = 1.104; MS Δ_{ST} = 1.182) and Lahn (mtDNA Δ_{ST} = 1.349; MS Δ_{ST} = 1.212) and lowest for the hatchery_2 stock.

Estimated genetic differentiation in terms of F_{ST} and Φ_{ST} were non-significant between most populations. Significant differentiations ranged from F_{ST} = 0.015 (NI vs. MU) to F_{ST} = 0.147 (DO vs. NI) at the microsatellite level and from Φ_{ST} = 0.118 (NI vs. MU) to Φ_{ST} = 0.942 (DO vs. H2) at the sequence level (Tab. 6). The only population from upstream the Rhine Falls, the Dornbirner Ach, was considerably more strongly differentiated from any of the other populations than the others were amongst each other. Assignment test indicated high self-assignment at the microsatellite level only for the populations Lahn (45.24%), Nister (47.06%), hatchery_1 (65.00%), hatchery_2 (78.00%) and Dornbirner Ach (100%). Correct self-assignment of individuals from the remaining populations ranged between 10–23% (Tab. 7).

Overall number of migrants was N_m = 6.47 (data not shown), indicating recent gene flow between many populations of the different tributaries of the Rhine. Highest N_m were observed between the northern Ketscher and the Lahn (N_m = 5.85), and the northern Ketscher and the Nister (N_m = 6.57) populations. However, N_m was explicitly lower between the Dornbirner Ach and all other investigated populations below the Rhine Falls and thus, gene flow from below the Rhine falls to the Constance/Alpine Rhine system is very unlikely. GeneClass2 identified seven and two first-generation migrants using the Lhome and Lhome/Lmax statistics, respectively (Tab. 7). These migrants were detected in the Lahn (N = 2), Murg (N = 3), northern Ketscher (N = 3), and Minthesee (N = 1).

Fixation indices were slightly negative in all populations, except for the Lahn where the highest value (F = 0.006) was obtained (Tab. 2). Hence, no evidence for significant inbreeding was observed at population level.

Estimated effective population size of N_e = 7.5 (CI: 3.5–13.3) was found for hatchery_2 stock. For all other populations N_e -values ranged from 78.6 (H1) to 364.3 (NK), whereas the populations Nister, Dornbirner Ach, Murg and Minthesee showed infinite N_e -values (data not shown). Infinite N_e -values in some populations are probably due to limited sample size. Infinite estimates are usually interpreted as no evidence for any disequilibrium caused by genetic drift (see Waples and Do, 2010; Marandel *et al.*, 2019 for further interpretation of N_e estimates). However, obtained N_e -values are conforming to the tests for recent bottlenecks. Based on the microsatellite data, evidence for a recent bottleneck was only found in the hatchery_2 stock under both models and test statistics (Tab. 3). Instead, the Lahn and Dornbirner Ach displayed evidence of significant heterozygous deficiency under both models under the sign test. The Nister showed significant heterozygous deficiency only under the SMM in the sign test.

4 Discussion

We used two different molecular markers to investigate the genetic diversity and variability within the endangered fish species, *Chondrostoma nasus*, in the River Rhine district, Germany. Overall diversity was quite diverging between the

Table 6. Pairwise Φ_{ST} - (above the diagonal) and F_{ST} -values (below the diagonal) of the investigated *Chondrostoma nasus* populations (Pop). Significant values are in bold.

Pop	H2	LA	NI	DO	WI	MU	MI	NK	H1
H2	—	0.286	0.379	0.942	0.013	0.074	0.039	0.219	0.020
LA	0.063	—	0.043	0.648	0.109	0.031	0.094	−0.012	0.122
NI	0.049	0.028	—	0.532	0.181	0.118	0.182	−0.004	0.194
DO	0.131	0.112	0.147	—	0.846	0.806	0.726	0.649	0.825
WI	0.031	0.031	0.010	0.132	—	−0.058	−0.019	0.038	−0.064
MU	0.037	0.019	0.015	0.136	0.016	—	−0.019	−0.014	−0.048
MI	0.019	0.028	0.020	0.130	0.012	0.009	—	0.066	−0.001
NK	0.036	0.028	0.017	0.124	0.006	0.011	0.003	—	0.049
H1	0.044	0.065	0.048	0.142	0.034	0.039	0.046	0.028	—

Table 7. Results of the self-assignment test and first generation migrant (F0) analysis. Each row contains the individuals from one sampling location and the columns indicate the populations to which these individuals were assigned (highest likelihood). Total number of investigated individuals per population (Total N), percentage of correctly assigned individuals (Corr. Assign. %) and the number of first generation migrants with their most likely source population in brackets for the two tests statistics Lhome and Lhome/Lmax are given. For the first generation migrant analysis, both hatchery stocks (H1 and H2) were excluded from the analysis.

Population	H2	LA	NI	DO	WI	MU	MI	NK	H1	Total N	Corr. Assign (%)	Migrants (F0)	
												Lhome	Lhome/Lmax
H2	39	2	4	0	0	0	2	0	3	50	78.00	—	—
LA	3	19	3	1	4	4	2	4	2	42	45.24	1(MI), 1(WI)	—
NI	3	1	24	0	9	6	2	4	2	51	47.06	—	—
DO	0	0	0	14	0	0	0	0	0	14	100.00	—	—
WI	1	0	3	0	3	2	1	1	3	14	21.43	—	—
MU	2	0	1	0	2	3	5	0	0	13	23.08	1(NI)	1(NI), 1(WI)
MI	2	0	2	0	1	1	1	3	0	10	10.00	1(MU)	—
NK	5	4	5	0	8	4	2	10	5	43	23.26	2 (LA), 1(NI)	—
H1	3	2	1	0	3	1	0	4	26	40	65.00	—	—

individual populations. Ten out of 11 haplotypes recorded in total, of which seven were private haplotypes, were undescribed so far. This reflects the limited number of genetic studies of nase in Germany and entire Europe. These previously undetected haplotypes will be relevant for the further assessment of phylogenetic aspects of the species and are of concern for proper conservation strategies, not only within the Rhine basin but also at a larger scale. Furthermore, it will be relevant for further investigation of hybridisation of *Parachondrostoma toxostoma*, a threatened species endemic to France and Switzerland (Paz-Vinas *et al.*, 2013), and *C. nasus* being invasive in France and Italy (e.g. Costedoat *et al.*, 2007).

Overall, low to moderate levels of population differentiation were observed. Results confirm a genetically distinct population in the Dornbirner Ach only. Hence, the Rhine Falls act as a physical barrier and prevent genetic exchange even for migrating fish (Hudson *et al.*, 2014). Furthermore, the Dornbirner Ach was represented by a haplotype (Hap_8; Fig. 2) that was not found in any other population downstream the Rhine Falls. Fish have colonized Lake Constance from two different glacial refugia (Vonlanthen *et al.*, 2011), the Rhine and the Danube. Such a contact zone/co-occurrence of different phylogenetic lineages/clades was already detected e.g. for burbot, *Lota lota* (Barluenga *et al.*, 2006; Wetjen *et al.*, 2020)

in Lake Constance. This may similarly reflect the distinctiveness of the Dornbirner Ach population in this study. Equivalent results were also apparent when the Dornbirner Ach was compared to other populations from Switzerland (Hudson *et al.*, 2014). Specimens of the Dornbirner Ach were genetically even more similar to Danubian populations than to Rhine populations below the Rhine Falls (Vonlanthen *et al.*, 2011). Such populations deserve high conservation priority by themselves.

The nase seems to disperse across large geographic distances (e.g. LA and MI; F0 migrants = 1; F_{ST} = 0.028; Φ_{ST} = 0.094). This was already investigated for nase (> 400 km) and other cyprinids, e.g. *Leuciscus idus* (> 100 km) (Winter and Fredrich, 2003 and cited literature within) and is supported by the low, non-significant levels of genetic differentiation among sampling locations downstream the Rhine Falls. Additionally, it is confirmed by the self-assignment analysis. The majority of individuals of MI, MU, WI and NK were not assigned to their sampling population. A pronounced substructuring (despite DO and LA) of the populations was not present, and most genetic variation was found within rather than between populations. Gene flow exceeded by far the benchmark of one migrant per generation. Hence, long-distance dispersal prevents genetic drift and isolation as long as

migration is not obstructed. Previous studies likewise detected distinct genetic groups of nase only between different major river basins where admixture is naturally impossible *e.g.* between the Rhine and the current Danube basin (Hudson *et al.*, 2014) that were connected in former times. Consequently, the dramatic population declines of nase in the River Lahn and elsewhere in the recent past are likely an ecological consequence of habitat loss/changes and fragmentation leading to changes in demography, rather than a consequence of inbreeding and the loss of genetic variation alone.

The middle reaches of the River Lahn are about twice as wide as the upper River Lahn where runoff fluctuations are much greater. Hence, it is possible that the donor population was not locally sufficiently adapted for reintroduction in the upper Lahn. Therefore, artificial restoring of spawning grounds and their morphodynamics is important (*e.g.* Hauer *et al.*, 2007; Kitada *et al.*, 2019) in the upper River Lahn. Stable riverbed structures are needed for successful spawning. Furthermore, the permeability and oxygen supply of the interstitial are essential factors and higher fine sediment accumulation reduce successful hatching (Duerregger *et al.*, 2018; Nagel *et al.*, 2019). Discharge and water quantities differ within the River Lahn. Reduced input of gravel during floods, a deepened watercourse and a deficiency of lateral erosion result in a lack of suitable spawning grounds and growth habitats for nase in the upper River Lahn. Hence, in the context of the current EU-LIFE project further renaturation and habitat enhancement measures are implemented.

Moreover, a major point affecting the establishment of a self-reproducing population in the upper Lahn is the presence of weirs between the middle and upper River Lahn. Even though there have been several key improvements ensuring the passage for migrating fish in the tributaries of the River Rhine and the River Lahn itself, further removals or effective fish passages are needed. Migrating nase may not be able to pass several weirs upstream *e.g.* the “Nehmühle”, “Steinmühle”, “Am Grün”, and “Wehrda” weirs near Marburg (Fig. 1). When barriers remain impassable, nase may show atypical behaviour. Instead of migrating upstream, stocked fish may migrate further downstream to search for suitable spawning areas. Such a downstream migration was investigated in translocated nase in the River Amblève, Belgium (Ovidio *et al.*, 2016). This might be an additional reason why nase was not noticed in the upper Lahn in recent years. Furthermore, the significant heterozygous deficits of the Lahn population indicate a recent population size expansion or an influx of alleles from different gene pools (Luikart and Cornuet, 1998), probably because of asymmetric (downstream-biased) gene flow (Paz-Vinas *et al.*, 2013).

Reintroduction and stock enhancement are often seen as a means of recovering threatened or lost species populations. However, stocks that have been bred in captivity can have deleterious effects on wild populations (*e.g.* Araki *et al.*, 2007; Christie *et al.*, 2012). In general, donor populations of high genetic variation are suitable for reintroduction of a species in lost parts of a species' range (Drauch *et al.*, 2007; Forsman, 2014) since heterozygosity is often related to fitness (García-Navas *et al.*, 2014; Feiner *et al.*, 2017). Interbreeding of wild populations with hatchery fish that were hatchery-bred over multiple generations or are of a different gene pool may cause changes in the genetic structure of wild populations due to

genetic drift, loss of genetic variability, reduced effective population size, outbreeding depression and general reduced fitness (*e.g.* Araki *et al.*, 2007; Christie *et al.*, 2012; Naish *et al.*, 2013). Our results show that the diversity was comparatively lower in the H2 stock. Furthermore, this stock passed through a recent bottleneck and had a comparatively low effective population size, which in turn may result in inbreeding (Naish *et al.*, 2013) and reduced genetic variability. Since the H2 stock was created in 1984, adaptation to captivity might have led to a reduced fitness of the individuals when they were released to the wild (Frankham, 2005) into the upper River Lahn. All these named aspects make the H2 stock rather unsuitable as a donor population for reintroduction purposes.

Even though, the H2 stock originated initially from the River Nister itself, the Lahn was stocked with H2 individuals, and the geographical proximity of LA to NI is more pronounced compared to LA and any other investigated population, these three populations showed significant genetic differentiation in terms of F_{ST} and Φ_{ST} . If the existing Lahn population really consisted solely of the stocked H2 individuals, we would not have expected significant genetic differences to H2 and NI or would at least expect them to be more similar to each other than compared to the other populations further upstream the River Rhine. Increased genetic differentiation is predicted for declining, isolated populations. Therefore, genetic distance between LA and NI suggests that populations drifted apart and gene flow did not fully counterbalance this effect, which might be reinforced by migration barriers between the rivers. It is likely that the re-established Lahn population does not exclusively originate from former stocking activities but established from autochthonous remainders or a combination of both. Another option would be that the H2 stock was actually not solely established by individuals from the River Nister but was also influenced by another different genetic pool. Otherwise, effects of captive breeding (reduced effective population size, selection) and separate development (genetic drift) of the stocks since creation of the H2 stock in 1984 must have put forth these differences.

The LA population was one of the most diverse ones investigated in this study and exhibited a unique and healthy genetic signature. It is obvious that fish from above the Rhine Falls from Lake Constance should definitively not be used for stocking the River Lahn or other water bodies below the Rhine Falls. For future reintroduction, a high number of spawners, ideally from the water body itself should be used. Using specimens of the self-reproducing population of the middle reaches of the River Lahn ensures to maintain the genetic characteristics (*e.g.* private haplotypes and alleles) and allows for a similar spawning time of the individuals. Spawning time is an adaptation to the temperature regime and has a strong genetic component (Schneider, 2011). Individuals could then directly be released into the upper Lahn before spawning actually takes place. This was already tested for nase, where downstream populations are still abundant (Ovidio *et al.*, 2016), and would be an alternative to stocking with hatchery-bred fish.

To further increase the number of specimens for reintroduction, juveniles may be artificially cultured by stripping the spawning animals directly at the middle Lahn. Since 2017, this method is exclusively used for reintroduction

in the upper Lahn in the context of an EU-LIFE project (Hübner *et al.*, 2017). Fertilized eggs are then raised up to stock size at a hatchery farm before they are released into the upper Lahn.

5 Conclusion

Chondrostoma nasus is a highly specialized freshwater species with a pronounced migration behaviour and high gene flow within the River Rhine system. Overall, low to moderate levels of population differentiation were estimated and highest genetic variance was found within rather than between the investigated populations. Distinct populations were solely found where migration is interrupted e.g., in the Dornbirner Ach upstream the Rhine Falls. However, individual populations (e.g. the River Lahn) downstream the Rhine Falls also account for specific genetic characteristics that should be regarded in future conservation strategies. Despite the limitation of an unambiguous determination of the origin of the existing nase population in the middle Lahn, several key recommendations can be derived from the results of this study to guide the restoration of the nase in the upper Lahn and/or other waters of the River Rhine basin. For a successful re-establishment of a self-reproducing nase population in the upper River Lahn on the long term, ideally spawners from the middle reaches of the water body itself should be used. However, it is not only important to carefully choose genetically sufficient stocking material, but also to restore habitats for spawning and growth, and to enhance river connectivity. Considering these aspects will allow for an establishment of a population robust enough in size and sufficiently diverse.

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